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A CD4$^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis

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Induction and maintenance of peripheral tolerance are important mechanisms to maintain the balance of the immune system. In addition to the deletion of T cells and their failure to respond in certain circumstances, active suppression mediated by T cells or T-cell factors has been proposed as a mechanism for maintaining peripheral tolerance[1]. However, the inability to isolate and clone regulatory T cells involved in antigen-specific inhibition of immune responses has made it difficult to understand the mechanisms underlying such active suppression. Here we show that chronic activation of both human and murine CD4$^+$ T cells in the presence of interleukin (IL)-10 gives rise to CD4$^+$ T-cell clones with low proliferative capacity, producing high levels of IL-10, low levels of IL-2 and no IL-4. These antigen-specific T-cell clones suppress the proliferation of CD4$^+$ T cells in response to antigen, and prevent colitis induced in SCID mice by pathogenic CD4$^+$CD45RB^{high} splenic T cells. Thus IL-10 drives the generation of a CD4$^+$ T-cell subset, designated T regulatory cells 1 (Tr1), which suppresses antigen-specific immune responses and actively downregulates a pathological immune response *in vivo*.

Immune tolerance towards self antigens is dependent on the ability of the immune system to discriminate between self and non-self[2,3]. This occurs mainly through clonal deletion in the thymus of self-reactive T lymphocytes at an early stage of development. However, the immune system is also exposed to extrathymic self antigens and to repetitive stimulation by non-pathogenic antigens through inhalation and ingestion of foreign substances. To avoid chronic cell activation and inflammation, the immune system must develop unresponsiveness to such stimuli. Several mechanisms of peripheral tolerance have been proposed, including T-cell anergy[4,5], T-cell deletion[2], and active immune suppression[1]. Understanding these mechanisms of tolerance induction is clinically relevant for the treatment of autoimmune diseases and in transplantation, where the graft must ultimately be recognized as self.

The induction of anergy[5] and cell deletion by apoptosis[2] have been documented both *in vitro* and *in vivo*, but the analysis of active immune suppression mediated by T cells has been hampered by the inability to generate and clone these cells *in vitro*. The importance of cytokines in the development of specialized Th cells is now clear. Th1 cells are induced by activation in the presence of IL-12, whereas IL-4 drives the differentiation of Th2 cells[6]. Here we show that repetitive stimulation of CD4$^+$ T cells in the presence of IL-10 induces the differentiation of a unique subset of T cells with immunoregulatory properties.

Ovalbumin (OVA)-specific naive CD4$^+$ T cells obtained from the $\alpha\beta$ T-cell antigen receptor (TCR) DO11-10 transgenic mice[7] repeatedly stimulated with splenic antigen-presenting cells (APCs) and OVA peptide in the presence of IL-10, or IL-4 and IL-10, displayed a cytokine profile distinct from that of the classical Th0, Th1 or Th2 phenotype[8]. These T cells produce high levels of IL-10 and IL-5 and low levels of IL-2 and IL-4 (Fig. 1). Another characteristic of these T-cell populations is that they proliferated poorly in response to antigenic stimulation (Fig. 2a). In contrast, CD4$^+$ T cells isolated from OVA-specific TCR transgenic mice repeatedly stimulated with OVA peptide in the presence of IL-4 alone displayed the typical Th2-type profile, secreting IL-4, IL-5 and IL-10 (Fig. 1)[9].

Analysis of T-cell clones isolated from the mouse CD4$^+$ T-cell populations that were repeatedly stimulated with OVA peptide and splenic APCs in the presence of IL-10 showed that half of the CD4$^+$ T-cell clones obtained displayed this cytokine profile. They produced high levels of IL-10 and undetectable levels of IL-2 and IL-4, whereas the levels of production of interferon (IFN)-γ and transforming growth factor (TGF)-β were comparable with those of Th0 and Th1 clones, respectively (Table 1). These T-cell clones also proliferated poorly in response to antigen-specific stimulation (Fig. 2a), which may explain the previous failure to isolate these cells. Only Th1, Th2 and Th0 clones were isolated from T-cell populations cultured in the absence of IL-10 (Table 1). Thus chronic activation of mouse CD4$^+$ T cells in the presence of IL-10 results in the generation of a T-cell subset with a unique cytokine profile and low proliferative capacity. These cells are designated Tr1 cells on the basis of the their function.

Tr1 cells can also be generated from human peripheral blood. Both alloantigen-specific CD4$^+$ T-cell clones (JDV24) and non-alloantigen-specific T-cell clones (JDV15, JDV308 and JDV94)

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displaying the Tr1 cell cytokine profile were derived from $CD4^+$ T-cell populations that had initially been stimulated with allogeneic monocytes in the presence of IL-10 (Table 2). In contrast, only Th1-type clones were obtained when the $CD4^+$ T cells were stimulated by allogeneic monocytes in the absence of IL-10. The most striking characteristic of the human Tr1 cell clones was their unusually high level of IL-10 production (Table 2). They also produced very low levels of IL-2, and failed to secrete detectable levels of IL-4, whereas their levels of IL-5, IFN-γ and TGF-β production were similar to those of human Th0 clones. Taken together, these results demonstrate that antigen-specific Tr1 cells can be generated *in vitro* from naive populations of both human and mouse $CD4^+$ T cells.

Human Tr1 clones stimulated with either immobilized anti-CD3 and anti-CD28 monoclonal antibodies or allogeneic monocytes (for JDV24) showed low proliferative responses that were sustained by secretion of IL-2, as the addition of an anti-IL-2 monoclonal antibody completely blocked the growth of the Tr1 cells (Fig. 2b). Kinetic studies showed that IL-10 is produced rapidly after activation of the human Tr1 clones. IL-10 is detectable in the supernatants of Tr1 clones 8 h after activation (data not shown), which indicates that IL-10 production by Tr1 clones generally occurs before or at the same time as IL-2 production. In contrast, production of IL-10 by Th0, Th1 and Th2 clones established from the same donor in the same experiment was not detectable until 20 h after activation, which is compatible with previous findings[10].

Early endogenous IL-10 production accounts in part for the low proliferative capacity of the human and mouse Tr1 clones in response to TCR/CD3 stimulation (Fig. 2), as proliferation of the

Table 1 Cytokine production by murine OVA-specific $CD4^+$ T-cell clones

T-cell clones	Initial incubation with IL-10	IL-2 (pg ml^{-1})	IL-4 (pg ml^{-1})	IL-10 (pg ml^{-1})	IFN-γ (ng ml^{-1})	TGF-β (pg ml^{-1})	Clone type
A-10-1	yes	<40	<50	1,230 ± 310	105 ± 27		Tr1
A-10-9	yes	<40	<50	1,020 ± 120	87 ± 17	435 ± 9	Tr1
A-10-11	yes	<40	<50	1,850 ± 410	217 ± 68	747 ± 9	Tr1
A-10-14	yes	<40	<50	1,820 ± 120	198 ± 67		Tr1
A-10-2	yes	102 ± 26	250 ± 45	850 ± 130	489 ± 124		Th0
A-10-7	yes	97 ± 27	897 ± 102	410 ± 120	267 ± 247		Th0
A-10-15	yes	52 ± 19	89 ± 17	205 ± 80	420 ± 41		Th0
A-10-19	yes	69 ± 27	890 ± 48	310 ± 120	107 ± 57		Th0
A-3	no	<40	1,247 ± 157	510 ± 110	<75		Th2
A-5	no	<40	879 ± 87	310 ± 60	<75		Th2
A-7	no	129 ± 48	<50	<75	375 ± 97	715 ± 2	Th1
A-13	no	89 ± 28	598 ± 59	215 ± 30	143 ± 13		Th0
A-14	no	203 ± 49	<50	<75	249 ± 49		Th1

Cytokine production by murine OVA-specific $CD4^+$ T-cell clones derived from T cells of DO11-10 TCR-transgenic mice repetitively stimulated in the presence or absence of IL-10. Naive (MEL14 bright) $CD4^+$ T cells from DO11-10 transgenic mice were stimulated with OVA peptide (0.6 μM) in the presence (A-10-) or absence (A-) of IL-10 (100 U ml^{-1}). After repetitive stimulations under the same conditions, the $CD4^+$ T cells from these two populations were cloned by limiting dilution. After expansion, the T-cell clones were stimulated by OVA peptide (1 μM) and irradiated total splenic APCs. Cytokines were analysed by ELISA in culture supernatants collected after 48 or 72 h of culture for TGF-β. Results represent pooled data from 4 representative experiments.

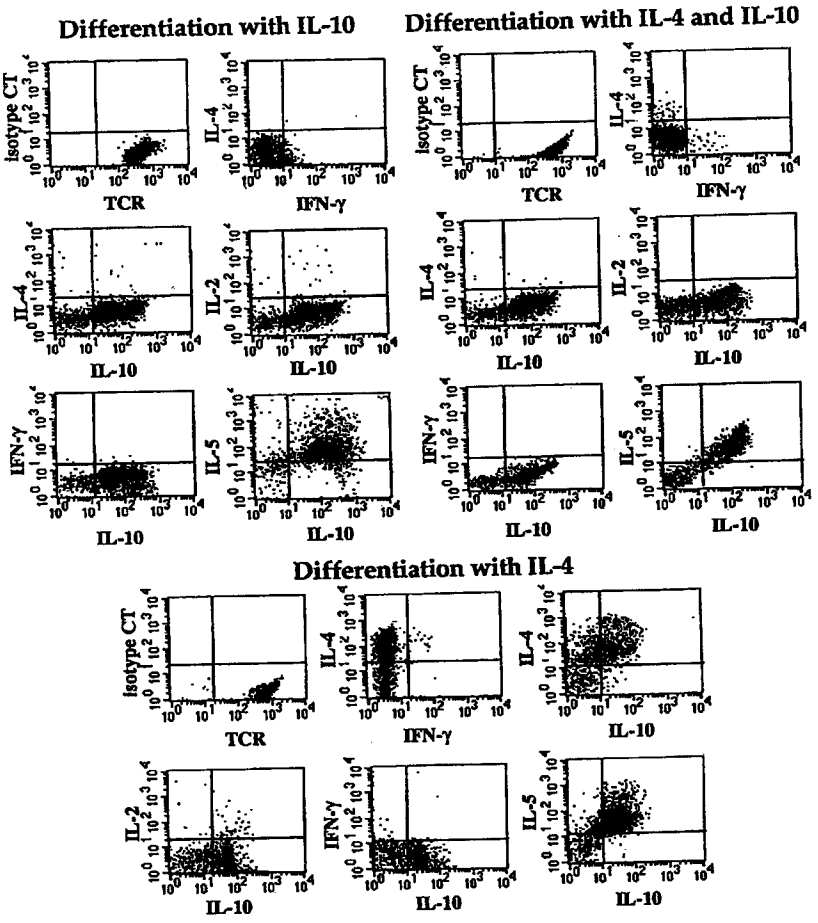

Figure 1 Generation of murine Tr1 cells in the presence of IL-10. Naive (MEL14 bright) $CD4^+$ T cells from BALB/c mice transgenic for the DO11.10 αβ TCR[7] were cultured with OVA peptide (0.6 μM) and irradiated splenic APCs in the presence or absence of: IL-4 (200 U ml^{-1}), IL-10 (100 U ml^{-1}), or both. The stimulation, under the same conditions, was repeated weekly for three consecutive weeks[9]. Cells were collected, washed and restimulated with immobilized anti-CD3 (10 μg ml^{-1}) and anti-CD28 mAb (10 μg ml^{-1}) for 4 h at 37 °C. Brefeldin A (10 μg ml^{-1}) was added for the last 2 h of culture[29]. The cells were fixed and stained for detection of intracellular cytokines using FITC or PE-conjugated monoclonal antibodies specific for IFN-γ, IL-2, IL-4, IL-5 and IL-10, or an isotype-matched control mAb (isotype CT), as indicated. Cells were also stained with the anti-TCR clonotype-specific mAb KJ1-26 (TCR) to show that all cells were positive for the transgene after 3 weeks of culture. One of three experiments is shown.

Tr1 clones was augmented by the addition of a neutralizing anti-IL-10 monoclonal antibody, which is consistent with previous observations indicating that IL-10 prevents or inhibits T-cell proliferation[11]. In contrast, anti-IL-10 monoclonal antibody had no effect on the proliferative responses of control Th1, Th2 or Th0 clones (Fig. 2). Proliferation of both human and mouse Tr1 clones was also partly augmented by a neutralizing anti-TGF-β monoclonal antibody (Fig. 2), whereas the proliferation of the control Th clones was unaffected (Fig. 2). The combination of anti-IL-10 and anti-TGF-β antibodies had additive effects and almost completely restored the proliferative responses of Tr1 clones (Fig. 2).

The observation that Tr1 cells secrete high levels of the immunosuppressive cytokine IL-10, and low levels of the T-cell growth-promoting cytokines IL-2 and IL-4, suggested that antigen-specific activation of Tr1 cells may result in inhibition of antigen-specific proliferation of other T cells. Indeed, co-culture experiments using a transwell system confirmed this notion for both human and mouse T cells. Resting human $CD4^+$ T cells were stimulated with irradiated allogeneic monocytes in the bottom compartment, whereas alloantigen-specific Tr1 clone JDV24 or non-antigen-specific Tr1 clone JDV308 were stimulated with the same allogeneic monocytes in the top compartment. Alloantigen-specific stimulation of the Tr1 clone JDV24 strongly reduced the alloantigen-specific proliferative responses of autologous human $CD4^+$ T cells, whereas the non-alloantigen-specific Tr1 clone JDV308 was ineffective (Fig. 3a). A slight increase in $CD4^+$ T-cell proliferation was noticed following co-culture with the alloantigen-specific T-cell clone JDV305, which belongs to the Th1 subset (Fig. 3a).

Similar data were obtained with mouse Tr1 clones (Fig. 3b). The proliferation of naive $CD4^+$ T cells in response to OVA peptide and splenic APCs was dramatically reduced following co-culture in the transwell system with OVA peptide-activated Tr1 clones or with activated Tr1-cell populations generated in the presence of IL-10. In contrast, OVA-specific Th0 clones or Th2-cell populations generated in

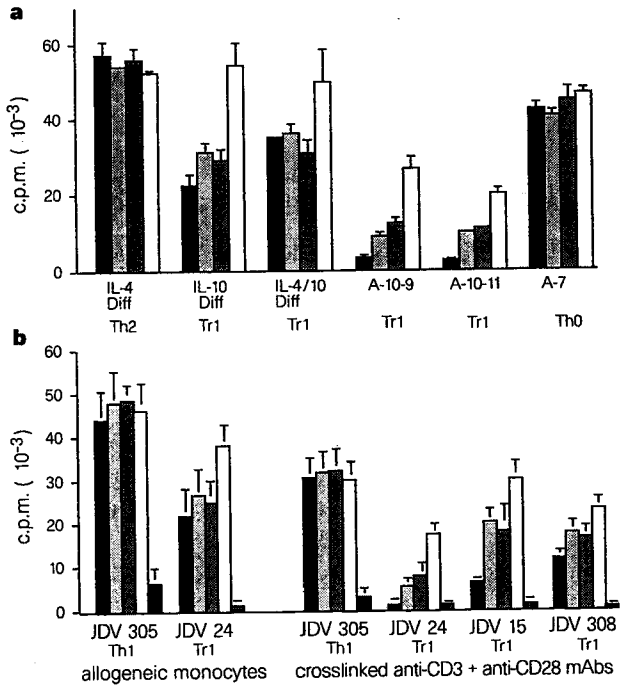

Figure 2 Proliferative responses of Tr1 cells. **a**, Naive (MEL14 bright) $CD14^+$ T cells from BALB/c mice transgenic for the DO11.10 αβ TCR[7] were differentiated (Diff) *in vitro* as described in Fig. 1 and restimulated with OVA peptide (0.6 μM) and irradiated splenic APCs for 3 days. One OVA-specific Th0 clone (A-7) and two OVA-specific Tr1 type clones (A-10-9) and A-10-11) were stimulated using the same conditions. **b**, The human Th1 (JDV 305) and Tr1 (JDV24) alloantigen-specific $CD4^+$ T-cell clones or the non-alloantigen-specific Tr1-cell clones (JDV15 and JDV 308) were stimulated with either allogeneic irradiated monocytes for 5 days or with immobilized anti-CD3 (10 μg ml^{-1}) and anti-CD28 mAb (1 μg ml^{-1}) for 3 days. The proliferative responses were measured by ^{3}[H]-TdR incorporation during the last 12 h of culture. Cultures of human or mouse T cells were carried out in the presence of an isotype-matched control mAb (GL113, rat IgG1 at 20 μg ml^{-1} + mouse IgG1 from Caltag (Burlingam, CA) at 20 μg ml^{-1}; black bars); a neutralizing anti-IL-10 mAb (12G8 at 5 μg ml^{-1} for human T cells or JES5-2A5 at 10 μg ml^{-1} for mouse T cells; white hatched bars); a neutralizing anti-TGF-β (from Genzyme (Cambridge, MA) at 10 μg ml^{-1}; black hatched bars); a combination of the two antibodies (white bars); or a neutralizing anti-IL-2 mAb (BG-5, 10 μg ml^{-1} for human cells; grey bars). A representative Th1 (JDV 305) and a representative Tr1(JDV24) are shown for antigen-specific response. One of four experiments is shown.

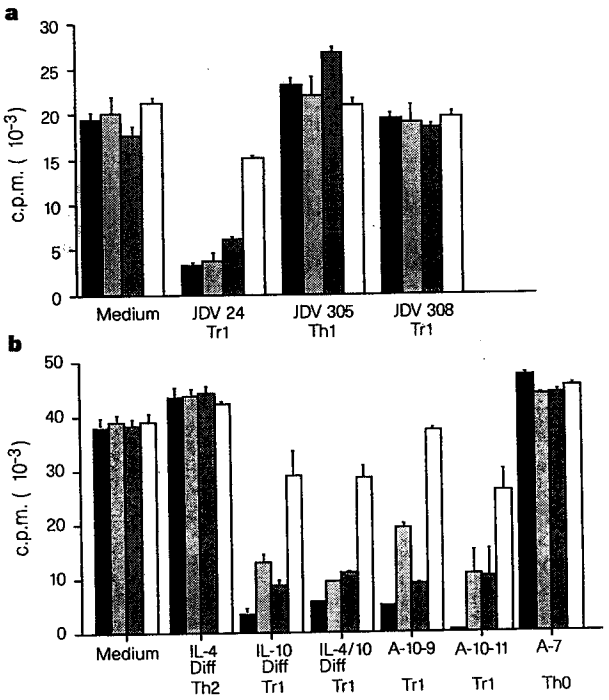

Figure 3 Tr1 cells inhibit the antigen-specific proliferative responses of naive $CD4^+$ T cells. **a**, Purified human $CD4^+$ T cells from donor JDV were stimulated by allogeneic purified irradiated monocytes for 5 days in the bottom compartment of a transwell system. The resting T cells were co-cultured with various autologous T-cell clones contained in the upper transwell compartment; the Tr1 clone JDV 24 and the Th1 clone JDV 305 are alloantigen-specific, whereas the Tr1 clone JDV 308 is non-alloantigen-specific. After 5 days the basket was removed and the proliferative response of the $CD4^+$ T cells was analysed by ^{3}[H]-TdR incorporation during the last 12 h of culture. **b**, Naive (MEL14 bright) $CD4^+$ T cells from BALB/c mice transgenic for the DO11.10 αβ TCR were stimulated in the bottom compartment of a transwell system with OVA peptide (0.6 μM) and irradiated APCs for 3 days. These naive T cells were co-cultured with the indicated populations contained in the upper transwell basket: OVA-specific $CD4^+$ T-cell populations differentiated (diff) *in vitro* by three consecutive restimulations in the presence of IL-4 (IL-4 Diff), IL-10 (IL-10 Diff) or IL-4 and IL-10 (IL4/10 Diff), respectively, or OVA-specific $CD4^+$ T-cell clones A-10-9, A-10-11 (Tr1 type clones) or A-7 (Th0 type clone). After 3 days the basket was removed and the proliferative response of the naive $CD4^+$ T cells was analysed by ^{3}[H]-TdR incorporation during the last 12 h of the culture. Cultures of human and murine cells were carried out in the presence of an isotype-matched control (GL113, rat IgG1 at 20 μg ml^{-1} + mouse IgG1 from Caltag Laboratories (Burlingame, CA) at 20 μg ml^{-1}; black bars); a blocking anti-IL-10 mAb (12G8 at 5 μg ml^{-1} for human cells or JES5-2A5 at 10 μg ml^{-1} for mouse cells; white hatched bars); a blocking anti-TGF-β (from Genzyme (Cambridge, MA) at 10 μg ml^{-1}; black hatched bars); or a combination of the two antibodies (white bars). Results represent one of three experiments performed.

Table 2 Cytokine production by human Tr1 cells

T-cell clones	Clone type	IL-2	IL-4	IL-5	IL-10	IFN-γ	TGF-β
Cells preincubated with IL-10							
JDV15	Tr1	<20	<40	5,210	11,620	1,370	220
JDV24	Tr1	<20	<40	12,540	14,370	420	230
JDV308	Tr1	70	<40	8,450	12,630	720	510
JDV94	Tr1	<20	<40	6,870	12,470	1,110	
JDV81	Th0	420	2,140	6,250	510	1,680	
JDV6	Th1	640	<40	90	720	6,480	
JDV110	Th1	420	<40	<40	640	4,290	
Cells preincubated without IL-10							
JDV305	Th1	140	<40	1,220	450	2,520	220
JDV5	Th1	850	<40	<40	1,120	8,450	
JDV40	Th1	480	<40	210	720	4,240	
JDV60	Th1	570	<40	330	440	3,590	
JDV101	Th1	440	<40	150	320	6,540	

To generate antigen-specific T-cell clones, $CD4^+$ T cells from donor JDV cells were stimulated with irradiated purified allogeneic monocytes in the presence or absence of IL-10 (100 U ml^{-1}). After 10 days, $CD4^+$ T cells were cloned. The different T-cell clones were expanded and selected for their specificity against the same allogeneic monocytes. To measure cytokine production, T cells were activated with crosslinked anti-CD3 (10 μg ml^{-1}) and anti-CD28 (1 μg ml^{-1}) mAbs, supernatants were collected after 48 h of culture or 72 h for TGF-β. Results are expressed in pg ml^{-1} and represent pooled data from four independent experiments.

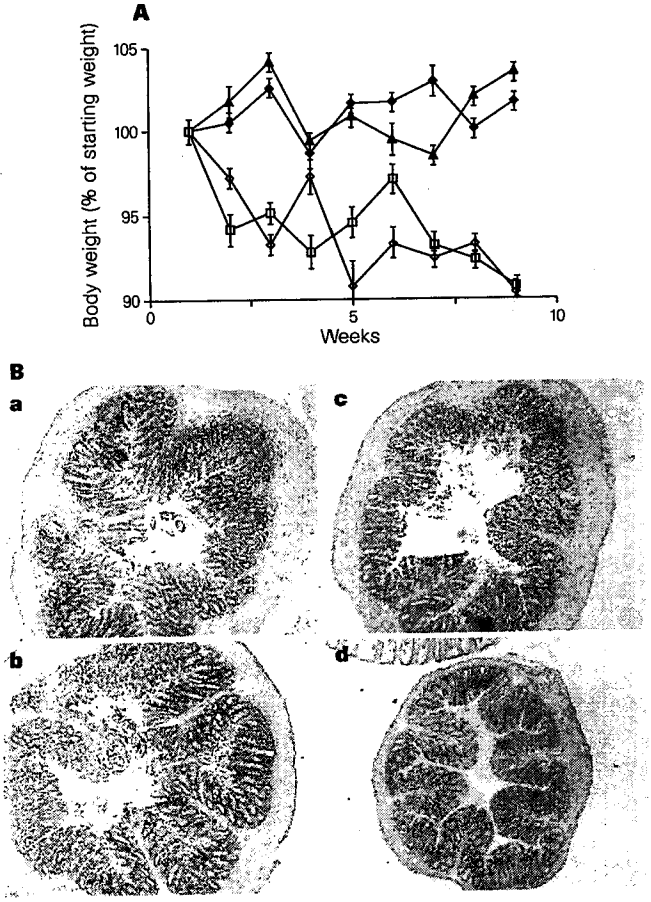

Figure 4 Co-transfer of Tr1 clones prevents colitis. **A**, Groups of 5 *scid* mice were reconstituted with 4×10^5 $CD4^+CD45RB^{hi}$ splenic T cells from 6-week-old BALB/c mice either alone (white squares) or in the presence of either 2×10^5 $CD4^+CD45RB^{lo}$ T cells (black triangles) or 2×10^5 OVA-specific Tr1 clones (A-10-9) (white diamonds for animal not fed with ovalbumin, and black diamonds for animals fed with ovalbumin). Half of the groups were fed with ovalbumin diluted in the drinking water (100 ng ml^{-1}). Feeding with ovalbumin did not change the pathogenic effect of $CD4^+CD45RB^{hi}$ T cells or the protective effect of $CD4^+CD45RB^{lo}$ T cells, and only animals fed with ovalbumin are shown for these two groups. Data represent mean plus s.e.m. of 5 animals per group. Results are representative of two experiments. **B**, Severe colitis in mice restored with $CD4^+CD45RB^{hi}$ T cells (**a**) and with $CD4^+CD45RB^{hi}$ T cells and a Tr1 clone (A-10-9) but not fed with ovalbumin (**b**). Protection from colitis in mice reconstituted with either $CD4^+CD45RB^{hi}$ T cells plus $CD4^+CD45RB^{lo}$ T cells (**c**) or in mice reconstituted with $CD4^+CD45RB^{hi}$ T cells and a Tr1 clone and fed with ovalbumin (**d**).

the presence of IL-4 had no suppressive effects, but rather enhanced OVA-induced proliferation of naive $CD4^+$ T cells.

Addition of neutralizing anti-IL-10 and anti-TGF-β antibodies augmented the proliferative responses of naive human and mouse $CD4^+$ T cells in the presence of Tr1 cells (Fig. 3). These data demonstrate that IL-10 induces the *in vitro* differentiation of a new regulatory $CD4^+$ T-cell subset, which suppresses antigen-specific T-cell responses in both mice and man. These suppressive activities are predominantly mediated by IL-10 and TGF-β. Moreover, similar to the data previously reported for T cells stimulated in the presence of exogenous IL-10 (ref. 12), naive T cells stimulated in the presence of Tr1 clones fail to proliferate and to secrete IL-2, IL-4, IL-5 and IFN-γ after re-stimulation (not shown).

We sought to establish whether these Tr1 clones were functional *in vivo* and could regulate a pathogenic T-cell response. IL-10 is important for the maintenance of T-cell tolerance in the gut, as IL-10-deficient mice develop inflammatory bowel disease, which is thought to be mediated by normal enteric antigens[13]. In addition, administration of IL-10 to young IL-10-deficient mice prevents inflammatory bowel disease[14]. IL-10 also significantly inhibits the development of inflammatory bowel disease (IBD) induced by the transfer of $CD4^+CD45RB^{hi}$ $D4^+$ T cells into SCID mice[15]. Here we show, in this latter model of IBD induction, that transfer in SCID mice of as few as 2×10^5 OVA-specific Tr1 cells (A-10-9 (Fig. 4) or A-10-11 (data not shown)) prevents IBD induced by pathogenic $CD4^+$ $CD45RB^{hi}$ splenic T cells. Not surprisingly, the Tr1 cells are effective only upon stimulation *in vivo* by feeding the mice with OVA (Fig. 4). No difference in the pathogenicity of the $CD4^+$ $CD45RB^{hi}$ splenic T cells or in the protective effects of the $CD4^+$ $CD45RB^{lo}$ T cells[16] was observed after feeding the mice with OVA in the absence of Tr1 cells (Fig. 4). These results demonstrate that Tr1 clones can prevent a T-cell-mediated disease *in vivo*. Furthermore, they indicate that the Tr1 cells must be activated *in vivo* to be effective. Finally, because the function of Tr1 cells is mediated by soluble factors, the present findings suggest that they can suppress active immune responses to unknown antigens in the microenvironment by an antigen-driven bystander suppression mechanism.

Transfer of $CD4^+$ T-cell clones isolated from the gut of SJL mice after oral tolerance induction with myelic basic protein (MBP)[17] or from the pancreas of NOD mice[18] has been shown to suppress experimental autoimmune myelitis and diabetes, respectively. These inhibitory activities were relieved by anti-TGF-β, suggesting that these clones mediated their suppression through TGF-β[17,18]. Although these T-cell clones appear to have similar functions to Tr1 cells, their cytokine production profiles are different. The T-cell clones isolated from SJL and NOD mice produce low levels of IL-10 and high levels of biologically active TGF-β, whereas the T-cell clones isolated from MBP transgenic mice fed with MBP (called Th3

cells) exclusively produced active TGF-β and no IL-10, IL-4, IL-2 or TNF-α[19]. In contrast, Tr1 cells consistently produce low levels of active TGF-β and high levels of IL-10. Another difference is that both cytokines contribute to the suppressive activity of Tr1 cells. Taken together, these data suggest that Th3 and Tr1 cells are distinct types of $CD4^+$ regulatory T cells.

The isolation of Tr1 cells from the peripheral blood of SCID patients, in which high levels of IL-10 *in vivo* are associated with tolerance after allogeneic stem-cell transplantation[10], and from anergic T cells stimulated by major histocompatability complex (MHC) class II-positive melanoma cells[20], supports the hypothesis that Tr1 cells are generated *in vivo* by chronic exposure of naive T cells to alloantigens in the presence of IL-10. Furthermore, based on the *in vitro* immunosuppressive activities of Tr1 cells, it is possible that these Tr1 cells specific for the host antigens have similar properties *in vivo*, and therefore may be responsible for transplantation tolerance in man. The observation that IL-10 production is associated with tolerance in murine models of organ and stem-cell transplantation is consistent with this notion[21,22].

However, administration of IL-10 failed to induce T-cell tolerance in other experimental models of transplantation[23–26] and autoimmune disease, such as experimental autoimmune encephalomyelitis[27] or autoimmune haemolytic anaemia[28], which could be explained by our observations suggesting that the potential induction of tolerance through differentiation of Tr1 cells is a lengthy process *in vivo*, requiring chronic antigen-specific stimulation in the presence of IL-10. Thus systemic administration of IL-10 after the onset of the disease may not impede the effector phase of the immune response, and may therefore not result in tolerance.

Our findings indicate that stimulation of $CD4^+$ T cells in the presence of IL-10 generates a subset of $CD4^+$ T cells that inhibits antigen-specific immune responses through the secretion of IL-10 and TGF-β. The cytokine profile appears to be stable, as Tr1 clones cultured for more than 12 months produced the same cytokine profile after activation. All regulatory cells described previously have been isolated after *in vivo* priming with antigens. The ability to generate Tr1 cells after exposure to antigen *in vitro* may be an advantage for further functional analysis and potential clinical applications, particularly because we have demonstrated their regulatory capacity *in vivo*. Moreover, because Tr1 cells suppress immune responses directed against other antigens in the microenvironment, they could potentially be used to regulate T-cell-mediated disease, even when the pathogenic antigen is unknown. □

Methods

Cloning of Tr1 cells. Human Tr1 cells were generated from peripheral blood $CD4^+$ T cells stimulated with allogeneic monocytes in the presence of exogenous IL-10. After 10 days, the $CD4^+$ T cells were cloned by flow cytometry. Cells were labelled with an anti-CD4-FITC monoclonal antibody (Becton Dickinson, Mountain View, CA) and the viable cells were sorted at one cell per well in wells pre-coated with anti-CD3 monoclonal antibody (SPV-T3 (ref. 12) 100 μg ml^{-1} in 100 μl Tris, 0.1 M, pH 9.4). After sorting, a mixture of irradiated feeder cells (JY, 10^5 per ml and PBMC, 10^6 per ml) and 10 U ml^{-1} of rIL-2 in 100 μl were added. Clones were expanded with IL-2 (10 U ml^{-1}) and by repetitive stimulation with high doses of crosslinked anti-CD3 monoclonal antibody (100 μg ml^{-1}). The cloning of the mouse Tr1 cells was performed by limiting dilution at 0.3 cells per well in wells coated with 50 μg ml^{-1} of anti-CD3 monoclonal antibody (2C11, PharMingen, San Diego) in PBS and containing irradiated total splenocytes (10^7 per ml) and 20 U ml^{-1} IL-2. Clones were expanded by culture with IL-2 (20 U ml^{-1}) and IL-4 (20 U ml^{-1}), OVA peptide (2 μM) and irradiated splenic APCs (10^7 per ml).

Flow cytometry analysis of intracellular cytokines. Analysis of intracellular cytokines by flow cytometry was performed as described[29]. Cells (10^6 per ml) were activated with immobilized anti-CD3 and anti-CD28 monoclonal antibodies (PharMingen, San Diego) for 4 h. Brefeldin A (Sigma, St Louis) was added at 10 μg ml^{-1}, and 2 h later cells were collected, washed and fixed with formaldehyde (2%). For intracellular staining, cells were incubated with the following monoclonal antibodies (PharMingen, San Diego): anti-IL-4-FITC or PE (11B11, 5 μg ml^{-1}), anti-IFN-γ-PE (XMG1.2, 5 μg ml^{-1}), anti-IL-5-PE (TRFK5-2, 5 μg ml^{-1}), anti-IL-10-FITC (JES-16E3, 5 μg ml^{-1}), anti-IL-2-PE (JES6-5H4, 2.5 μg ml^{-1}) or the anti-clonotype KJ1-26-FITC (5 μg ml^{-1}). Samples were analysed on a FACScan (Becton Dickinson, Mountain View, CA).

Cytokine analysis. Human $CD4^+$ T-cell clones or populations at 1×10^6 per ml were stimulated by immobilized anti-CD3 (10 μg ml^{-1}) and anti-CD28 monoclonal antibodies (1 μg ml^{-1}) for 48 h. The production of IL-2, IL-4, IL-5, IL-10 and IFN-γ was measured by immunoenzymatic assays, as described[12]. The amounts of IL-2, IL-4, IL-10 and IFN-γ produced by murine $CD4^+$ T-cell clones was measured by ELISA in supernatants collected 48 h after culture of the T cells (10^6 per ml) stimulated with OVA-peptide (1 μM) and irradiated total splenic APCs, as described[9]. For TGF-β measurements, cells were cultured for 72 h in Yssel's medium[30] without serum and the amount of TGF-β was measured by ELISA (R and D System, Minneapolis, MN) after an acid activation, according to the manufacturer's instructions.

Proliferation assays. All proliferation assays were carried out in Yssel's medium[30] supplemented with 10% FCS and 1% human serum for human cells or with 10% FCS for murine cells. Alloantigen-specific stimulation was performed by culturing human $CD4^+$ T-cell clones (10^6 per ml) with purified irradiated (4,000 rad) monocytes (10^6 per ml) in 200-μl round-bottomed 96-well plates (Linbro, ICN Biomedicals, Aurora, OH). T-cell proliferation was measured after 5 days incubation at 37 °C and 5% CO_2 and a 12-h pulse with ^{3}[H]-TdR. Activation of human $CD4^+$ T cells with immobilized anti-CD3 and anti-CD28 mAb was performed as described[12]. In brief, antibodies were diluted at the indicated concentration in 0.1 M Tris buffer, pH 9.4, and plates incubated for a week at 4 °C. After washing the plates, $CD4^+$ T cells were added at a concentration of 5×10^4 cells per well and proliferation was measured after 3 days by ^{3}[H]-TdR uptake. Similarly, murine $CD4^+$ T-cell populations or T-cell clones were stimulated at a concentration of 5×10^4 cells per well with OVA peptide (0.5 μM) and irradiated (4,000 rad) total splenocytes (5×10^5 cells per well) for 3 days.

Inflammatory bowel disease. The induction of IBD was performed as described[16]. In brief, C.B-17 *scid* mice 8–12 weeks old. (Simonsen Laboratories, Gilroy, CA) were injected intraperitoneally with 100 μl of PBS containing 4×10^5 $CD4^+CD45RB^{hi}$ sorted splenic T cells with or without either 2×10^5 $CD4^+CD45RB^{lo}$ splenic T cells or 2×10^5 Tr1 clones. Half of the mice were fed with OVA diluted in the drinking water at 100 ng ml^{-1}. Weight gain or loss, as a percentage of original starting weight, was scored weekly for 9 weeks. For preparation of tissue for histopathologic analysis, approximately 10-mm segments of the distal large intestine were removed and fixed in a solution of 10% formalin. Fixed tissues were frozen and sections were prepared and stained with haematoxylin.

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Lipopolysaccharide-binding protein is required to combat a murine Gram-negative bacterial infection

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An invading pathogen must be held in check by the innate immune system until a specific immune response can be mounted. In the case of Gram-negative bacteria, the principal stimulator of the innate immune system is lipopolysaccharide (LPS), a component of the bacterial outer membrane[1]. *In vitro*, LPS is bound by lipopolysaccharide-binding protein (LBP)[2] and transferred to CD14—the LPS receptor on the macrophage surface[3,4]—or to high-density lipoprotein (HDL) particles[5,6]. Transfer to CD14 triggers an inflammatory response which is crucial for keeping an infection under control. Here we investigate how LBP functions *in vivo* by using LBP-deficient mice. Surprisingly, we find that LBP is not required *in vivo* for the clearance of LPS from the circulation, but is essential for the rapid induction of an inflammatory response by small amounts of LPS or Gram-negative bacteria and for survival of an intraperitoneal *Salmonella* infection.

The mouse LBP gene was disrupted in embryonic stem cells by homologous recombination. The mutant cells were injected into BALB/c blastocysts and a resulting mosaic male was selected to generate heterozygous and homozygous LBP-deficient animals (Fig. 1). Serum from animals heterozygous for the mutation contained LBP detectable by enzyme-linked immunosorbent assay (ELISA), but the serum from homozygous knockout mice did not (Table 1). LBP function was measured as the capacity to transfer LPS labelled with fluorescein isothiocyanate to mouse CD14 expressed on the surface of CHO cells; this was readily demonstrated in $LBP^{+/-}$ and $LBP^{+/+}$ sera but none is detectable in the serum of the $LBP^{-/-}$ homozygotes (Fig. 2). By these criteria, homologous recombination has resulted in a complete loss of LBP activity in our homozygous mutant animals.

Both heterozygous and homozygous LBP-deficient animals appear normal and are fertile. A preliminary analysis of tissue from all main organs of male and female $LBP^{-/-}$ mice at 14 weeks of age revealed no differences compared with an $LBP^{+/-}$ age-matched littermate. LBP may be involved in lipid transfer reactions[7], so we determined the concentrations of total serum cholesterol and triglycerides in heterozygous and homozygous animals: the

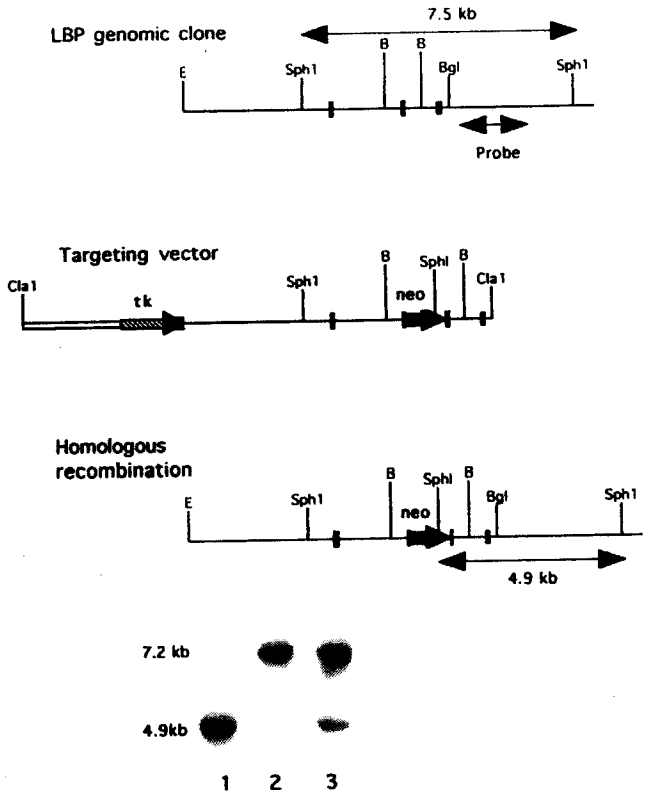

Figure 1 Maps of the mouse genomic clone containing three exons of the LBP gene (top), the targeting vector (middle) and the expected structure of the recombinant chromosome (lower). Southern analysis of three F_2 littermates: homozygous LBP-deficient animal (lane 1), wild-type (lane 2) and heterozygote (lane 3).